

The University of Chicago

STUDIES ON THE EXTRACTION OF A  
PRECIPITABLE SUBSTANCE FROM  
THE GENUS BRUCELLA

A DISSERTATION SUBMITTED TO THE  
FACULTY OF THE DIVISION OF THE BIO-  
LOGICAL SCIENCES IN CANDIDACY FOR  
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

1933

By  
DOROTHY OLIVE REITER

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# STUDIES ON THE EXTRACTION OF A PRECIPITABLE SUBSTANCE FROM THE GENUS BRUCELLA

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CHICAGO

The genus *Brucella*, or *Alcaligenes*, is composed of three commonly recognized varieties or species, the *melitensis*, the *abortus*, and the *suis*. These varieties, although closely related morphologically, biochemically, and serologically, differ somewhat in glucose, nitrogen, and sulfur metabolism, sensitivity to dyes, and pathogenicity.

McAlpine and Slanetz<sup>1</sup> studied the metabolic activity of representative cultures and found that *abortus* strains of bovine origin could be separated from *abortus* strains of porcine and human origin and from *melitensis* strains on the basis of their nitrogen and glucose metabolism. Huddleson<sup>2</sup> separated the genus into three distinct groups or species according to the time and rate of H<sub>2</sub>S production. McAlpine and Slanetz<sup>3</sup> have shown that CO<sub>2</sub> stimulates the growth of the bovine strains, but partially inhibits the growth of the other varieties.

Differences have been observed among the strains when grown on the various dye mediums; the dye plate method of Huddleson<sup>2</sup> or the semi-solid method of Meyer and Zobell.<sup>4</sup> By their growth differences on these mediums, strains can be classified into three varieties, the *melitensis*, the *abortus*, and the *suis*.

Slight differences have been observed in the pathogenicity of the various strains for monkeys. Fleischner, Vecki, Shaw, and Meyer<sup>5</sup> found *melitensis* strains more pathogenic than *abortus* strains. Huddleson and Hallman<sup>6</sup> observed that *suis* strains were the most invasive and like Fleischner and his collaborators reported that *melitensis* strains were more pathogenic than those of the *abortus* variety.

Various serological studies have been made of the members of this genus. By direct agglutination no appreciable differences are observed between the varieties but, by agglutination absorption the genus can be divided into two varieties, the *melitensis* and the *abortus*; agglutination absorption does not differentiate the bovine and porcine varieties. Reciprocal agglutination absorption studies show the close relationship of the strains. Evans<sup>7</sup> found that

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1. *J. Infect. Dis.* **42**: 66 and 73, 1928.

2. Undulant Fever Symposium, New York, American Public Health Association, 1929, p. 18.

3. *J. Infect. Dis.* **43**: 232, 1928.

4. *J. Infect. Dis.* **51**: 72, 1932.

5. *J. Infect. Dis.* **29**: 663, 1921.

6. *J. Infect. Dis.* **45**: 293, 1929.

7. Studies on *Brucella* (*Alcaligenes*) *Melitensis*, Hyg. Lab. Bull. 143, 1925.

strains of the abortus variety absorbed 87.5 per cent of the agglutinins from serum of the melitensis A variety and that the reciprocal relationship was the same. Futamura's<sup>8</sup> absorption tests likewise show the close relationship between the abortus and melitensis varieties.

Few attempts have been made to study the chemical nature of the varieties of the genus *Brucella*. Favilli and Biancalani<sup>9</sup> obtained polysaccharides of the immunological behavior of incomplete antigens from melitensis and paramelitensis strains, but failed to isolate a polysaccharide from the abortus strain. Their failure to isolate a polysaccharide from the abortus variety is interesting in view of the close morphological, biochemical, and serological relationship of the members of this genus.

Because of the close relationship between the varieties of the genus *Brucella*, a study was made of the alcohol insoluble antigenic substance of representative melitensis, abortus, and suis strains. The strains were obtained from the National Institute of Health through the courtesy of Dr. Geo. W. McCoy. Strains 456 and 428 have been used by the National Institute of Health as type strains of the abortus and melitensis varieties. Strain 41-1, porcine abortus, was more recently isolated.<sup>10</sup>

The strains were tested for their reaction on dye mediums, using Meyer's semi-solid method. Table 1 summarizes the results.

TABLE 1.—*Bacteriostatic Action of Dyes*

| Dye           | Strain |       |      |
|---------------|--------|-------|------|
|               | A456   | S41-1 | M428 |
| Fuchsin       | +      | 0     | +++  |
| Pyronine      | +++    | 0     | +++  |
| Thionine      | 0      | +++   | +++  |
| Methyl violet | 0      | 0     | +    |

+ = growth.  
0 = no growth.

The results of the direct agglutination tests using living antigens, organisms grown on glycerol liver infusion agar for forty-eight hours at 37 C., are summarized in table 2.

TABLE 2.—*Direct Agglutination Tests*

| Strain      | Dilution of serum |      |      |      |      |      | Control |
|-------------|-------------------|------|------|------|------|------|---------|
|             | 80                | 160  | 320  | 640  | 1280 | 2560 |         |
| Serum A456  |                   |      |      |      |      |      |         |
| A456        | ++++              | ++++ | ++++ | ++++ | +    | 0    | 0       |
| S41-1       | ++++              | ++++ | ++++ | ++++ | +    | 0    | 0       |
| M428        | ++++              | ++++ | ++++ | ++   | 0    | 0    | 0       |
| Serum S41-1 |                   |      |      |      |      |      |         |
| A456        | ++++              | ++++ | ++++ | ++++ | +++  | 0    | 0       |
| S41-1       | ++++              | ++++ | ++++ | ++++ | +++  | 0    | 0       |
| M428        | ++++              | ++++ | ++++ | +++  | +    | 0    | 0       |
| Serum M428  |                   |      |      |      |      |      |         |
| A456        | ++++              | ++++ | ++++ | ++   | ±    | 0    | 0       |
| S41-1       | ++++              | ++++ | ++++ | +++  | ±    | 0    | 0       |
| M428        | ++++              | ++++ | ++++ | +++  | 0    | 0    | 0       |

8. J. Japanese Soc. Vet. Sc. **3**: 127, 1924.

9. Sperimentale, Arch. di biol. **86**: 357, 1932.

10. These strains are designated respectively as A456 (abortus type), M428 (Melitensis), and S41-1 (suis).



Table 2 shows that strain M428 did not agglutinate to so high a titer as the other strains with the heterologous serums, and that it did not stimulate quite so rapid an immune response in animals. The animals all had been immunized in the same way.

This change in antigenicity is interesting, since the results of agglutination studies performed two years previously showed no difference in the height of the titer when this strain was tested with heterologous serums.

By agglutination absorption, strains A456 and S41-1 partially absorbed the agglutinins from serum M428 and completely exhausted A456 and S41-1 serums of agglutinins. Strain M428 likewise partially removed the agglutinins from serums A456 and S41-1 and completely exhausted serum M428 of agglutinins.

All the strains formed homogeneous suspensions in saline and did not spontaneously agglutinate.

#### MORPHOLOGICAL CHARACTERISTICS OF THE STRAINS

Due to the fact that two of the strains, A456 and M428, were older laboratory cultures, a study was made of the colony morphology of the organisms. Henry,<sup>11</sup> Gwatkin,<sup>12</sup> McAlpine, Plastring and Brigham,<sup>13</sup> and Plastring and McAlpine<sup>14</sup> have shown that variants occur in the brucella group which differ from the ordinary form in colony morphology, biochemical activity, glucose utilization, and serology.

The strains were grown in veal infusion broth and glycerol liver infusion broth, streaked on the corresponding agar mediums and the colonies examined with the colony microscope.

In veal infusion broth the strains caused a slight clouding of the medium with no surface ring or pellicle and slight or no sediment in forty-eight hours at 37 C. After incubation for two weeks at 37 C. the strains produced a slight ring and sediment.

In glycerol liver infusion broth the strains likewise caused an even clouding of the medium with slight or no sediment. After two weeks' incubation strains A456 and S41-1 produced a pellicle and a heavier viscid sediment. Strain M428 produced a ring and a slight sediment. In spite of the pellicle and sediment, the broth remained turbid.

Strains A456 and S41-1 on veal infusion agar and glycerol liver infusion agar produced colonies of practically uniform size, smooth, and glistening. Strain M428, however, produced colonies of two sizes, large and small. The smaller colonies were clearer and more transparent and could readily be observed only with a colony microscope after seventy-two hours' growth.

Transfers made from the larger colonies to veal infusion broth showed a slight clouding in twenty-four hours. Transfers made from the smaller colonies seldom showed an appreciable clouding before forty-eight hours. Transfers from such broth cultures to veal infusion agar or glycerol liver infusion

11. Proc. Soc. Exper. Biol. & Med. **26**: 101, 1928.

12. Canad. Pub. Health J. **23**: 485, 1933.

13. J. Infect. Dis. **45**: 485, 1929.

14. J. Infect. Dis. **46**: 315, 1930.

agar showed no marked differences in colony size after forty-eight hours' incubation. However, when transfers were made to veal infusion agar or glycerol liver infusion agar slants, incubated forty-eight hours, kept in the ice box for a short time and replated, two colony types were again discernible. The colony types did not seem to be stable forms, but rather seemed to revert from one to the other upon subsequent transfer. The larger and smaller colonies were tested bacteriostatically, but no differences were observed in their reactions on the dye mediums. The strain was not spontaneously agglutinable, was serologically active, and was therefore used for extraction purposes. Several more recently isolated strains of the different varieties were likewise grown in broth and streaked on veal infusion agar and glycerol liver infusion agar. Colonies of two sizes were observed. McAlpine, Plastridge and Brigham<sup>13</sup> likewise observed large and small colonies on liver infusion agar. Henry<sup>11</sup> reported the occurrence of two colony types, the opaque and the transparent, on plates made from twenty-three stock cultures.

#### EXTRACTION OF PRECIPITABLE SUBSTANCE

The method used for extracting a precipitable substance from the varieties of the genus *Brucella* was similar to that used by Furth and Landsteiner<sup>15</sup> in obtaining a precipitable substance from the bacilli of the salmonella group and consisted of alcohol and saline extraction, followed by precipitation of the saline soluble constituents with an excess of 95 per cent alcohol. Favilli and Biancalani,<sup>9</sup> in obtaining the polysaccharides from the brucella group, also used alcohol and saline extraction, followed by precipitation of the saline soluble constituents with alcohol. Their material from the paramelitensis strain was purified by the addition of trichloroacetic acid followed by precipitation with absolute alcohol from an alkaline solution. The polysaccharide from the melitensis strain was purified by precipitation with uranium acetate followed by precipitation with alcohol.

#### METHOD OF EXTRACTION USED IN THIS WORK

The organisms were grown on glycerol liver infusion agar prepared, with slight modifications, according to the method of Huddleson, Hasley, and Torrey.<sup>16</sup> In order to simplify the preparation of the medium, the albumin precipitation was omitted, the broth was filtered only through tightly packed glass wool, and five per cent glycerol and Merck's finely powdered agar were added. The medium was relatively clear and gave an abundant growth of the organisms.

Petri dishes were inoculated with the growth from forty-eight hour glycerol liver infusion agar slants. Approximately eighty to one hundred plates were inoculated with each strain. After incubation at 37 C. for seventy-two to eighty-four hours the bacterial mass was obtained by emulsifying the growth in physiological salt solution. The bacteria were washed with saline and centrifuged, and 300-400 cc. of 95 per cent alcohol was added to the residue and placed in the ice box for twelve to eighteen hours.

15. J. Exper. Med. **49**: 727, 1929.

16. J. Infect. Dis. **40**: 352, 1927.



The centrifuged bacterial mass was boiled with 250–300 cc. of absolute alcohol under a reflux for one hour and filtered hot through a hot water filter. The extracts from all the strains filtered readily, and the filtrates usually remained clear even after standing at room temperature or at ice box temperature for several days.

The bacterial residue was extracted with 300–400 cc. of 75 per cent alcohol for two hours on a water bath and filtered hot through a hot water filter. The material usually filtered slowly, although differences were observed with the various strains: for example, strains A456 and S41–1 filtered readily and yielded a clear filtrate, whereas M428 filtered slowly and yielded an opalescent filtrate.

The bacterial residue which remained after absolute alcohol and 75 per cent alcohol extraction was boiled with 400–500 cc. of 0.9 per cent sodium chloride for two hours, centrifuged, and the slightly opalescent supernatant was evaporated by boiling to about three-fourths of its volume. The material when cold was precipitated with normal hydrochloric acid and centrifuged. The acid was added drop by drop until further addition no longer brought down a precipitate. The white flocculent precipitate yielded a strongly positive biuret and xanthoproteic reaction and precipitated homologous and heterologous immune serums in low dilutions, 1:50, 1:100 up to 1:500. No differences were observed in the height of the titer or in the amount of the precipitate.

To the clear supernatant, obtained after the protein precipitate had been removed by centrifugalization, N/10 HCl and N/10 NaOH were added drop by drop in order to make sure that the optimum precipitation point had not been passed. Any precipitate that formed was removed by centrifuging. The solution was alkalinized by the addition of 1/10 the volume of normal NaOH and precipitated with four volumes of absolute alcohol. Precipitation was complete in twenty-four to forty-eight hours. The material, after being washed several times with 95 per cent alcohol, was dried with absolute alcohol and ether.

#### CHEMICAL CHARACTERISTICS OF THE ALCOHOL PRECIPITABLE SUBSTANCE

The dried precipitable substance from all the strains was a white powder soluble in water. The substances gave a negative xanthoproteic reaction and were not precipitated by any of the substances used to precipitate proteins, such as picric acid, tannic acid, or trichloroacetic acid. The substances gave a negative biuret test when 2 cc. of a 10 per cent NaOH solution was mixed with 2 cc. of a concentrated solution of the precipitable substance and 0.1 per cent  $\text{CuSO}_4$  solution was added. When different concentrations of the reagents were used different results were obtained. For example, when 1 cc. of a saturated solution of NaOH was added to 4 cc. of a concentrated solution of the precipitable substance, mixed, and 2 drops of diluted Fehling's solution A was added,  $2\frac{1}{2}$  cc. of Fehling's solution diluted with 10 cc. of distilled water, the precipitable substances from the strains A456 and S41–1 gave a slightly positive test while the extract from strain M428 gave a nega-

tive test. The material when dissolved in water did not reduce Fehling's solution. After hydrolysis with N/3 HCl for five hours extracts from the three varieties strongly reduced Fehling's solution.

Repeated attempts to further purify the material by precipitation with trichloroacetic acid and picric acid from acid and alkaline solutions failed. Alcohol precipitation from acid and neutral solutions also was unsuccessful.

#### IMMUNE REACTIONS OF THE PRECIPITABLE SUBSTANCE

Rabbits were immunized with organisms grown in liver infusion broth and on liver infusion agar slants for forty-eight hours. The growth from the broth cultures was centrifuged, washed with saline, and adjusted to a density of 2 on McFarland's nephelometer. The growth from the agar slants was emulsified in saline, centrifuged, and the sediment adjusted to a density of 2. The antigens were heated at 60 C. for forty minutes and tested for sterility in liver infusion broth and on glycerol liver infusion agar.

Rabbits were immunized by injecting 2 cc. of the heated vaccines intravenously. They were bled from the heart on the sixth day after the injection, reinjected on the following day, and again bled from the heart twelve to fourteen days later.

Broth and agar antigens were used in order to study their effect on the precipitation reaction. The broth antigens were used in order to eliminate cross reactions due to anti-agar immune substances. In 1931, Sordelli and Mayer<sup>17</sup> showed that serums prepared by immunizing animals with agar cultures of *B. anthracis* or *Bact. typhosum* contained precipitins for agar. Zozaya and Medina<sup>18</sup> confirmed these findings and indicated that false cross agglutination and precipitation might occur when agar cultures were used for immunization and for the preparation of test suspensions or extracts.

#### PRECIPITATION TEST

The antigen used in the precipitation test was a saline solution of the dried precipitable substance. A weighed amount of the material was dissolved in saline and quantitative dilutions were prepared. In the test, 0.2 cc. of the saline solution of the antigen was mixed with 0.2 cc. of immune serum, incubated for two hours at 37 C. in a water bath, and placed in the ice box for twelve to eighteen hours. Readings were taken after ice box incubation with artificial light against a dark background.

The results of the precipitation tests are summarized in table 3.

The alcohol insoluble substances extracted from the abortus, the suis, and the melitensis varieties of the genus *Brucella* precipitated homologous and heterologous immune serums. Considered from the standpoint of precipitability of abortus and suis immune serums, the strains fall into three varieties. Arranged in the order of antigenic activity they are: the abortus, precipitable in dilutions of 1:50,000, the suis, precipitable in dilutions of 1:10,000 to 1:25,000, and the melitensis, precipitable in dilutions of 1:1000 to 1:5000.

17. *Compt. rend. Soc. de biol.* **107**: 736, 1931; **108**: 675, 1931

18. *J. Exper. Med.* **57**: 41, 1933.



TABLE 3.—*Precipitation Tests*

| Serum         | Precipitable substance | Dilution |      |      |      |        |        |        |
|---------------|------------------------|----------|------|------|------|--------|--------|--------|
|               |                        | 500      | 1000 | 2500 | 5000 | 10,000 | 25,000 | 50,000 |
| A456 (broth)  | A456                   | ++++     | ++++ | ++++ | ++++ | ++++   | +++    | +      |
|               | S41-1                  | ++++     | ++++ | ++++ | ++++ | +      | 0      | 0      |
|               | M428                   | +++      | ++   | +    | 0    | 0      | 0      | 0      |
| S41-1 (broth) | A456                   | ++++     | ++++ | ++++ | ++++ | ++++   | ++     | +      |
|               | S41-1                  | ++++     | ++++ | ++++ | ++++ | +      | +      | 0      |
|               | M428                   | ++++     | +++  | ++   | +    | 0      | 0      | 0      |
| M428 (broth)  | A456                   | ++++     | ++   | +    | 0    | 0      | 0      | 0      |
|               | S41-1                  | ++++     | +    | ±    | 0    | 0      | 0      | 0      |
|               | M428                   | ++++     | +++  | +    | ±    | 0      | 0      | 0      |
| A456 (agar)   | A456                   | +++      | ++++ | ++++ | ++++ | ++++   | ++     | +      |
|               | S41-1                  | +++      | ++++ | ++++ | ++++ | +++    | +      | 0      |
|               | M428                   | ++++     | ++++ | +++  | +    | 0      | 0      | 0      |
| S41-1 (agar)  | A456                   | ++++     | ++++ | ++++ | ++++ | ++++   | +++    | +      |
|               | S41-1                  | ++++     | ++++ | ++++ | ++++ | +++    | ++     | 0      |
|               | M428                   | +++      | +    | 0    | 0    | 0      | 0      | 0      |
| M428 (agar)   | A456                   | +++      | +    | 0    | 0    | 0      | 0      | 0      |
|               | S41-1                  | +++      | +    | 0    | 0    | 0      | 0      | 0      |
|               | M428                   | ++++     | +++  | ++   | +    | 0      | 0      | 0      |

In *Br. melitensis* var. *melitensis* antiserum no such differences are observed. In general the precipitating titer is low for all strains and is slightly higher with the homologous than with the heterologous precipitable substance.

The abortus variety not only precipitates its own serum in high dilutions, but also precipitates heterologous suis serum in higher dilutions than the suis precipitable substance. The abortus extract is active in the same dilution, 1:50,000, in both abortus and suis antisera. The suis extract is likewise active, with one exception, to approximately the same titer, 1:25,000, in both abortus and suis antisera.

Serums from two other strains, 481 and 89, were also used in precipitation tests. The characteristics of these strains may be briefly considered. Strain 481 was classified by Evans<sup>7</sup> as the only representative of the serological group to which it belonged. She observed that it was the only strain isolated in this country which precipitated spontaneously in saline. Because of its spontaneous agglutination it was difficult to carry out the simple agglutination tests, and its relationship to the other groups was established only by testing its property of absorbing the agglutinins from the various sera. The strain when used in these studies spontaneously agglutinated in saline, and no satisfactory bacterial suspension was obtained even by reducing the salt content to 0.2 to 0.4 per cent. Nine-day liver infusion broth cultures showed pellicle formation, a viscid sediment, and clearness of the broth. On the usual dye mediums growth was obtained only in thionin, indicating the strain to be of the porcine variety.

Strain 89 was classified by Francis,<sup>19</sup> in 1931, as of the *melitensis* variety. When used in the present studies, the strain was not agglutinated by A456, M428, or S41-1 antisera although it still gave the dye reactions of the *melitensis* variety. It caused an even clouding of liver infusion broth, was

readily emulsified in saline, and produced unusually small colonies on glycerol liver infusion agar.

The agglutinating titers of serums 481 and 89 are shown in tables 4 and 5. Living antigens were used and the tests were incubated at 56 C. for four hours and then placed in the ice box overnight.

Tables 4 and 5 show that strains A456, M428, and S41-1 either were not agglutinated or were agglutinated to only a low titer by the serums from strains 481 and 89. Strain 89 was likewise agglutinated in only a comparatively low titer.

TABLE 4.—*Serum 481*

| Antigen | Dilution of serum |      |      |     |     |     | Control |
|---------|-------------------|------|------|-----|-----|-----|---------|
|         | 20                | 40   | 80   | 160 | 320 | 640 |         |
| A456    | ±                 | 0    | 0    | 0   | 0   | 0   | 0       |
| M428    | ++++              | +    | ±    | 0   | 0   | 0   | 0       |
| S41-1   | ++                | +    | 0    | 0   | 0   | 0   | 0       |
| 89      | ++++              | ++++ | ++++ | +++ | +   | 0   | 0       |

TABLE 5.—*Serum 89*

| Antigen | Dilution of serum |      |    |     |     |     | Control |
|---------|-------------------|------|----|-----|-----|-----|---------|
|         | 20                | 40   | 80 | 160 | 320 | 640 |         |
| A456    | ±                 | 0    | 0  | 0   | 0   | 0   | 0       |
| M428    | ++++              | ++   | ±  | 0   | 0   | 0   | 0       |
| S41-1   | ++++              | ++   | ±  | 0   | 0   | 0   | 0       |
| 89      | ++++              | ++++ | ++ | +   | 0   | 0   | 0       |

Table 6 shows the results of precipitation tests obtained with serums 481 and 89 and the precipitable substance from strains A456, S41-1, and M428.

TABLE 6

| Serum | Precipitable substance | Dilution |      |      |      |        |        |        |        |
|-------|------------------------|----------|------|------|------|--------|--------|--------|--------|
|       |                        | 500      | 1000 | 2500 | 5000 | 10,000 | 25,000 | 50,000 | 75,000 |
| 481   | A456                   | ++++     | ++++ | ++++ | ++++ | ++     | ++     | 0      | 0      |
|       | S41-1                  | ++++     | ++++ | ++++ | ++++ | ++     | +      | 0      | 0      |
|       | M428                   | ++++     | ++   | +    | 0    | 0      | 0      | 0      | 0      |
| 89    | A456                   | +++      | ++++ | ++++ | ++++ | ++++   | ++++   | ++     | 0      |
|       | A41-1                  | ++++     | ++++ | ++++ | ++++ | ++++   | +      | 0      | 0      |
|       | M428                   | ++++     | ++++ | ++++ | ++++ | ++     | 0      | 0      | 0      |

The precipitation tests indicate that strains 481 and 89 produced an immune response in rabbits which could be demonstrated when an alcohol precipitable substance was used as an antigen, but which was not readily demonstrable when the whole bacteria were used as antigens. It seems that the precipitable substance is a more sensitive antigen than the bacteria.

#### SPECIFICITY OF THE PRECIPITABLE SUBSTANCE

Reference to table 3 indicates that practically no differences are observed in the precipitating titer of a serum or in the extent of the cross precipitation reactions when serums obtained by injecting organisms grown in broth or on agar are used. It seems that the cross reactions are due to a substance extracted from the bacteria rather than to the agar on which the bacteria were grown.



In order to learn something of the strain specificity of the precipitating substances, immune serums were absorbed with the extracts from strains A456, S41-1, and M428. In the first series of absorptions 1 cc. of immune serum was mixed with 1 cc. of a saline solution of the precipitable substance, incubated four hours at 37 C., and placed in the ice box overnight. The precipitate was removed by centrifuging and the serums diluted and used in the agglutination test.

The agglutinating titer of the absorbed serums was used as an index of the extent of absorption, because it is difficult to accurately read the precipitating titer when diluted serum is used. The results of the absorption tests are given in table 7.

TABLE 7.—*Absorption of Immune Serum with Precipitable Substances*

| Serum | Absorbed with | Dilution | Agglutination test |      |      |      |      |     |         |
|-------|---------------|----------|--------------------|------|------|------|------|-----|---------|
|       |               |          | Dilution           |      |      |      |      |     | Control |
|       |               |          | Antigen            | 20   | 40   | 80   | 160  | 320 |         |
| A456  | A456          | 1: 500   | A456               | ++++ | ++++ | ++++ | ++   | 0   | 0       |
|       |               |          | S41-1              | ++++ | ++++ | ++++ | +    | 0   | 0       |
|       |               |          | M428               | ++++ | +++  | +    | 0    | 0   | 0       |
|       | S41-1         | 1: 500   | A456               | ++++ | ++++ | +++  | +    | 0   | 0       |
|       |               |          | S41-1              | ++++ | ++++ | ++++ | ++   | 0   | 0       |
|       |               |          | M428               | ++++ | ++++ | ++   | 0    | 0   | 0       |
|       | M428          | 1: 250   | A456               | ++++ | ++++ | ++++ | +++  | ±   | 0       |
|       |               |          | S41-1              | ++++ | ++++ | ++++ | ++   | ±   | 0       |
|       |               |          | M428               | ++++ | ++++ | ++++ | +    | 0   | 0       |
| S41-1 | A456          | 1: 500   | A456               | ++++ | ++++ | +++  | ++   | ±   | 0       |
|       |               |          | S41-1              | ++++ | ++++ | ++++ | +    | ±   | 0       |
|       |               |          | M428               | ++++ | +++  | +    | 0    | 0   | 0       |
|       | S41-1         | 1: 500   | A456               | ++++ | ++++ | +++  | +    | 0   | 0       |
|       |               |          | S41-1              | ++++ | ++++ | ++++ | +    | 0   | 0       |
|       |               |          | M428               | ++++ | ++   | +    | 0    | 0   | 0       |
|       | M428          | 1: 250   | A456               | ++++ | ++++ | ++++ | ++++ | ++  | 0       |
|       |               |          | S41-1              | ++++ | ++++ | ++++ | ++++ | ++  | 0       |
|       |               |          | M428               | ++++ | ++++ | ++++ | +++  | +   | 0       |
| M428  | A456          | 1: 250   | A456               | +++  | +    | 0    | 0    | 0   | 0       |
|       |               |          | S41-1              | +++  | +    | 0    | 0    | 0   | 0       |
|       |               |          | M428               | +++  | ++   | ±    | 0    | 0   | 0       |
|       | S41-1         | 1: 250   | A456               | +++  | +    | 0    | 0    | 0   | 0       |
|       |               |          | S41-1              | +++  | +    | 0    | 0    | 0   | 0       |
|       |               |          | M428               | ++++ | ++   | ±    | 0    | 0   | 0       |
|       | M428          | 1: 250   | A456               | +++  | +    | 0    | 0    | 0   | 0       |
|       |               |          | S41-1              | +++  | ±    | 0    | 0    | 0   | 0       |
|       |               |          | M428               | ++++ | ++   | ±    | 0    | 0   | 0       |

The unabsorbed serums had an agglutinating titer of approximately 1:1280 (table 2). After absorption with the precipitable substances, the agglutinating titers were reduced; the extent of the reduction depending upon the substance used. For example, the extracts from strains A456 and S41-1 in 1:500 dilutions removed more antibodies from the corresponding serums than the extract from strain M428 in a 1:250 dilution. The agglutinating titer of the absorbed serum M428 was lower than that of the other serums; however, unabsorbed M428 serum also had a slightly lower titer than the other serums. Furthermore, more of the antibodies may have been removed because a lower dilution of the precipitable substance of strains A456 and S41-1 was used for absorbing the serum.

In order to determine whether the agglutinating titer could be further reduced by absorption with precipitable substance, serum was mixed with an equal amount of the precipitable substance (as in the previous tests), incubated at 37 C. for four hours, centrifuged, and 1 cc. of the substance was added to the supernatant. The serum was incubated for two hours at 37 C., placed in the ice box overnight, centrifuged, and used for the agglutination test.

No differences were observed in the agglutination results, whether the serums were absorbed once or twice with the precipitable substances.

In order to determine whether the reduction in the agglutinating titers was due to a non-specific change in the serum constituents, *S. schottmülleri* and *S. aertrycke* serums were absorbed with the precipitable substances from brucella strains. No reduction in the agglutinating titer was observed when the absorbed serums were tested with the homologous organisms.

Brucella antisera were also treated with the chemical precipitate produced by the addition of sodium hydroxide to absolute alcohol. No reduction in the agglutinating titer was observed.

These experiments indicate that the reduction of the agglutinating titer of brucella antisera is due to some substance specific for the genus, but not specific for the different varieties of the genus.

#### IN VIVO ANTIGENIC ACTIVITY OF THE PRECIPITABLE SUBSTANCE

Rabbits were injected intravenously with 2 cc. of a 1:200 dilution of the precipitable substances. Approximately 30 mg. of the extracts from strains A456, S41-1, and M428 were injected. The rabbits were bled six days after the last injection, and the serum was tested for precipitating and agglutinating antibodies. The results are summarized in tables 8 and 9.

TABLE 8.—*Precipitating Titer of Serum due to Injection of Precipitable Substance*

| Immunized with | Antigen | Dilution |       |     |      |
|----------------|---------|----------|-------|-----|------|
|                |         | 100      | 200   | 500 | 1000 |
| A456           | A456    | ++++     | +++   | +   | 0    |
|                | S41-1   | ++++     | +++++ | ++  | 0    |
|                | M428    | ++++     | +++   | +   | 0    |
| S41-1          | A456    | +++      | +     | 0   | 0    |
|                | S41-1   | ++++     | ++    | 0   | 0    |
|                | M428    | +++      | +     | 0   | 0    |
| M428           | A456    | +++      | +     | 0   | 0    |
|                | S41-1   | +++      | +++++ | +   | 0    |
|                | M428    | ++++     | +++   | +   | 0    |

Favilli and Biancalani<sup>9</sup> attempted to immunize rabbits with the purified material extracted from melitensis and paramelitensis strains. The serum of their animals did not show a precipitating titer when tested with the polysaccharides. They did not report agglutination or anaphylactic experiments.

The stimulation of antibodies in rabbits following the injection of precipitable substance may be due to the presence of a small residue of protein material. In order to determine whether the immune response was due to



the presence of protein material, guinea pigs were injected intravenously with 15 mg. of the precipitable substances dissolved in saline. Ten days later they were injected intravenously with 50 mg. of the same material.

The guinea pig injected with the extract from strain A456 showed a typical anaphylactic reaction, but did not die. The animal that received extract M428 showed only a slight reaction, difficulty in breathing, and roughness of the fur. In the case of the guinea pig sensitized with the S41-1 extract, an attempt was made to determine whether one-half of the shock dose (25 mg.) would cause an anaphylactic reaction. Only a slight reaction was obtained, roughness of the fur, urination, and slight difficulty in breathing. No further symptoms were observed after a second injection of 25 mg. about fifteen minutes later.

TABLE 9.—*Agglutinating Titer of Serum Due to Injection of Precipitable Substance*

| Immunized with | Antigen | Dilution |      |      |      |      |     | Control |
|----------------|---------|----------|------|------|------|------|-----|---------|
|                |         | 10       | 20   | 40   | 80   | 160  | 320 |         |
| A456           | A456    | ++++     | ++++ | ++++ | ++++ | ++++ | +++ | 0       |
|                | S41-1   | ++++     | ++++ | ++++ | ++++ | ++++ | +++ | 0       |
|                | M428    | ++++     | ++++ | ++++ | ++++ | ++++ | ++  | 0       |
| S41-1          | A456    | ++++     | ++++ | ++   | ±    | 0    | 0   | 0       |
|                | S41-1   | ++++     | ++++ | +    | ±    | 0    | 0   | 0       |
|                | M428    | ++++     | ++++ | +    | 0    | 0    | 0   | 0       |
| M428           | A456    | ++++     | ++++ | ++++ | ++   | +    | 0   | 0       |
|                | S41-1   | ++++     | ++++ | ++++ | ++   | +    | 0   | 0       |
|                | M428    | ++++     | ++++ | ++++ | ++   | +    | ±   | 0       |

Another explanation may be suggested to account for the immune response in animals. Zinsser<sup>20</sup> found that when alkaline extracts of pulverized bacteria of several varieties were precipitated with acid in the cold, boiled with acid, alkalinized to a  $p_H$  7, and again filtered, a small amount of an alcohol-precipitable material remained which reacted negatively to the biuret, Hopkins-Cole, Millon, and sulfosalicylic acid protein tests. Such filtrates, however, gave precipitation turbidity with ten volumes of absolute alcohol which varied in intensity in different preparations. He suggested that the alcohol-precipitable material was merely incidental, carrying down the antigenic material with it, since antigenic potency did not parallel alcohol precipitation. His material was not antigenic in animals. There is a possibility that the alcohol-precipitable material may be antigenic in the absence of protein material, if a sufficiently large molecule of the alcohol precipitate is obtained. Zozaya<sup>21</sup> has shown that polysaccharides can be rendered antigenic by haptogenic absorption upon a colloid carrier. He used collodion, aluminum hydroxide, casein, and bacterial cells as colloids, and polysaccharides from *B. anthracis*, the meningococcus, *Streptococcus viridans* (Bargen), *B. proteus*, *S. morgani*, *B. dysenteriae*, both the Shiga and Hiss type, and the pneumococci.

20. J. Exper. Med. **37**: 275, 1923.

21. J. Exper. Med. **55**: 325, 1932.

## DISCUSSION

While this investigation was in progress a paper appeared by Favilli and Biancalani<sup>9</sup> on the extraction of hydrocarbons from organisms of the brucella group. By using alcohol and saline extraction followed by alcohol precipitation, they obtained antigenic substances of the nature of haptenes from a melitensis and a paramelitensis strain, but not from an abortus strain. They suggested that their failure to isolate a carbohydrate from the abortus variety was due to the fact that in this strain the carbohydrate is more intimately linked with the bacterial protein.

In the work reported here preparations of a carbohydrate nature were extracted from the three varieties of the genus, the melitensis, the abortus, and the suis. Unlike the results obtained by the Italian workers, a carbohydrate substance was as readily extracted from the abortus variety as from the other two.

These preparations, like those of Favilli and Biancalani,<sup>9</sup> precipitated both homologous and heterologous brucella antisera. The preparation from the abortus strain was the most active, precipitating abortus and suis antisera in a dilution of 1:50,000. The suis extract precipitated in dilutions of 1:10,000 to 1:25,000. The melitensis extract precipitated melitensis antiserum in a dilution of 1:5000, abortus antiserum in dilutions of 1:2500 to 1:5000, and suis antiserum in dilutions of 1:1000 to 1:5000. The melitensis carbohydrate extracted by the Italian workers precipitated melitensis antiserum in a dilution of 1:800 and abortus antiserum in a dilution of 1:12,800. They also found, as I did, that melitensis serum was precipitated in relatively low dilutions by extracts of homologous and heterologous strains.

The polysaccharids extracted by Favilli and Biancalani<sup>9</sup> were of the nature of haptenes. The preparations obtained in this investigation stimulated the production of precipitating and agglutinating antibodies. Definitely positive precipitation tests, however, were obtained only when low dilutions of the precipitable substances were used (1:100 to 1:200). The agglutinating titers also were low, except in the case of the rabbit immunized with extract A456. This extract also was the only preparation which caused typical anaphylaxis in a guinea pig. The preparations from the other strains caused only a mild reaction characterized by slight difficulty in breathing. The antigenic response in animals may be due to the presence of a slight protein residue or may be due to the fact that the extracts were absorbed on a sufficient amount of an alcohol precipitable substance to render them antigenic. Zinsser<sup>20</sup> found that alcohol precipitable substances obtained from various bacteria were not antigenic in animals. His material, however, was precipitated at  $p_H$  7. It is possible that by increasing the alkali content of a solution a precipitate of larger molecular size is obtained which may be feebly antigenic.

The results of the precipitation tests using the substances extracted in this work indicate a closer relationship between the abortus and the suis varieties than between these and the melitensis variety. By agglutination absorption tests differences have likewise been observed between the meli-



tensis and the abortus varieties, but not between the bovine and the suis varieties.

There is a possibility that the differences between the three varieties of the genus *Brucella* may be due to their residence in different animal hosts. Theobald Smith<sup>22</sup> believed "that the porcine variety has been developed from cattle in recent times in the Middle West as a result of the very close association of the two species in the feed lots and the adaptation of the bovine variety to the pig under certain unknown conditions." Differences in the serological reactions of the organisms of the brucella group may also be caused by prolonged growth on artificial mediums. Plastringe and McAlpine<sup>14</sup> have shown that the mucoid variant is not agglutinated by antiserum prepared against the ordinary strains of the brucella organisms, but mucoid antiserum agglutinates both the mucoid variant and the ordinary strains, indicating that the mucoid variant possesses a double antigen complex, whereas only one complex is present in the normal form.

The method of chemical fractionation has been used in studying many groups of bacteria, yeasts, and fungi. Furth and Landsteiner<sup>15</sup> prepared specific precipitable substances from the main serological types of the typhoid-paratyphoid group and found that the specificity of the precipitin reactions of these substances paralleled in a general way the so-called small flaking agglutination. In some instances, as in the case of the pneumococci, highly type specific carbohydrates were obtained. In other instances, as in the case of the C substance of the pneumococci, group characteristics were found. Occasionally preparations from different species of organisms show chemical similarities, as in the case of the Friedländer's bacillus, the type II pneumococcus, and one variety of yeast.

In the brucella group, a group practically indistinguishable morphologically, serologically, biochemically, and pathogenically, the carbohydrate substance isolated from the three strains was specific for the species, but not for the varieties.

The results of the precipitation absorption tests seem to indicate that the differences between the preparations may be quantitative rather than qualitative, since the preparations from all the strains were able to reduce the agglutinating titers of the serums to about the same extent. If the alcohol precipitable material may be considered incidental carrying down the specific material with it, then differences in the amount of absorption might account for the differences in the activity of the preparations from the three varieties.

#### SUMMARY

Specific precipitable substances of polysaccharide nature were prepared from the three varieties of the genus *Brucella* by saline extraction, followed by precipitation with alcohol. These substances were feebly antigenic in rabbits, but failed to give any of the usual chemical tests for proteins with

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22. J. Exper. Med. 43: 207, 1926.

the exception that under certain conditions the substance from strains A456 and S41-1 gave a doubtfully positive biuret test. The specific substance from the abortus strain caused a typical anaphylactic reaction in a guinea pig when 15 mg. was used as a sensitizing dose. The specific substance from the melitensis and the porcine strains failed to give a typical reaction. The preparations from the three varieties precipitated brucella antiserums, but did not precipitate heterologous serums from the salmonella group. Serums absorbed with the precipitable substances from the three varieties showed a considerable reduction in the agglutinating titer of both homologous and heterologous brucella antiserums, indicating that the differences observed in the specific precipitable substances from the three strains may be quantitative rather than qualitative.

















